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Mechanism of sequence-specific template binding by the DNA primase of bacteriophage T7

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ABSTRACT

DNA primases catalyze the synthesis of the oligoribonucleotides required for the initiation of lagging strand DNA synthesis. Biochemical studies have elucidated the mechanism for the sequence-specific synthesis of primers. However, the physical interactions of the primase with the DNA template to explain the basis of specificity have not been demonstrated. Using a combination of surface plasmon resonance and biochemical assays, we show that T7 DNA primase has only a slightly higher affinity for DNA containing the primase recognition sequence (5'-TGGTC-3') than for DNA lacking the recognition site. However, this binding is drastically enhanced by the presence of the cognate Nucleoside triphosphates (NTPs), Adenosine triphosphate (ATP) and Cytosine triphosphate (CTP) that are incorporated into the primer, pppACCA. Formation of the dimer, pppAC, the initial step of sequence-specific primer synthesis, is not sufficient for the stable binding. Preformed primers exhibit significantly less selective binding than that observed with ATP and CTP. Alterations in subdomains of the primase result in loss of selective DNA binding. We present a model in which conformational changes induced during primer synthesis facilitate contact between the zinc-binding domain and the polymerase domain.

INTRODUCTION

The replication of a duplex DNA molecule is a complex process requiring an assembly of numerous proteins, the replisome (1,2). At the replication fork, DNA helicase

unwinds dsDNA to provide a single-stranded DNA (ssDNA) template on which DNA polymerase polymerizes nucleotides for leading strand DNA synthesis. The simultaneous synthesis of both DNA strands is intricate since the two strands have opposite polarities, yet DNA polymerases function in only one direction by adding nucleotides to the 3'-end of a growing chain. This problem is solved in most replication systems by a discontinuous mode of DNA replication whereby one strand, the leading strand, is synthesized continuously and the other strand, the lagging strand, is synthesized in small units. These Okazaki fragments are subsequently joined to yield a continuous lagging strand. Since DNA polymerase requires a preexisting oligonucleotide, the synthesis of each Okazaki fragment is dependent on a DNA primase that catalyzes the synthesis of an oligonucleotide designated as a primer. The oligoribonucleotides are extended by DNA polymerase to generate the Okazaki fragments.

The prokaryotic DNA primases—DnaG protein of *Escherichia coli*, gene 61 protein of bacteriophage T4 and gene 4 protein of bacteriophage T7 have been extensively studied (3). These DnaG-type primases all recognize a trinucleotide sequence in the template where they synthesize a dinucleotide that is then extended to yield oligonucleotides of 11, 5 or 4 nucleotides. The 3'-nucleotide in the trinucleotide of the template is 'cryptic' in that it is required for recognition but is not copied into the primer. These primases are composed of two distinct domains, a zinc-binding domain (ZBD) located at the N-terminus of the protein and an RNA polymerase domain (RPD) to which it is tethered by a flexible linker. The ZBD is responsible for recognition of the trinucleotide sequence in the template. The RPD contains an active site in which condensation of the NTPs is catalyzed by a two-metal mediated mechanism. Conserved amino acid residues are found in a DNA binding cleft composed of basic side

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chains (4) and in a TOPRIM-fold containing several catalytic acidic residues (5). The ZBD and RPD are connected by a flexible linker that facilitates interaction between the two domains (6). The C-terminal region of these primases contains an ancillary domain for association with the cognate helicase (7).

T7 DNA primase is encoded by gene 4 of the phage. The T7 DNA primase is unique in that the primase occupies the N-terminal half of the multifunctional 63-kDa gene 4 protein (8). The C-terminal half of the gene 4 protein encodes the T7 DNA helicase. The primase and helicase domains are connected via a flexible linker region that, together with N-terminal residues of the helicase domain, is essential for oligomerization of protein into a functional hexamer (9,10). Genetic manipulation allows for the individual expression of both domains of gene 4 protein, the resulting fragments having active helicase or primase (11,12). However, the tandem organization of the two activities bestows several advantages on the primase domain; it can use the translocation activity of the helicase to access primase recognition sites on DNA, the high affinity of the hexameric helicase for ssDNA stabilizes the primase at its recognition sites and the location of the primase places it in position to synthesize primers on the single-stranded lagging strand extruded by the helicase. The primase may also take advantage of its contact with the helicase to regulate the movement of the latter during primer synthesis. Leading strand synthesis pauses during the lengthy synthesis of a primer, thus providing a means for the coordination of leading and lagging strand synthesis (13). An interesting feature of the 63-kDa gene 4 protein is that a truncated form lacking the N-terminal ZBD of the primase domain (56-kDa form) is expressed in an equal amount to the full-length protein (14). The 56-kDa protein oligomerizes as a hexamer and has full helicase activity but lacks template-directed primer synthesis (15). The role of the shorter (56-kDa) gene 4 protein *in vivo* is not known.

The crystal structure of the primase domain alone (Figure 1A) as well as that of the 56-kDa gene 4 protein is available (6,16). However, there is not yet a structure of the primase in complex with ssDNA. Structural studies revealed that the ZBD is comprised of four β strands containing four cysteines coordinated with a zinc ion (6,17). Two subdomains in the RPD, TOPRIM-fold and the N-terminal part, form a basic cleft for template binding and a catalytic center, as suggested from mutational studies (18,19). The trinucleotide sequence recognized by T7 DNA primase is 5'-GTC-3' at which the dinucleotide pppAC is synthesized; the 3'-cryptic cytosine is not copied into the primer (Figure 1B). These dinucleotides cannot function as primers for the T7 DNA polymerase and require extension to a tetranucleotide. Provided the proper nucleotides are present in the expanded recognition site (5'-GGGTC-3', 5'-TGGTC-3' or 5'-GTGTC-3'), the resulting primers (pppACCC, pppACCA or pppACAC, respectively) are extended by T7 DNA polymerase. Preformed di-, tri- and tetranucleotides can substitute for the synthesis of the respective oligonucleotide (20). While preformed di- and trinucleotides need to be

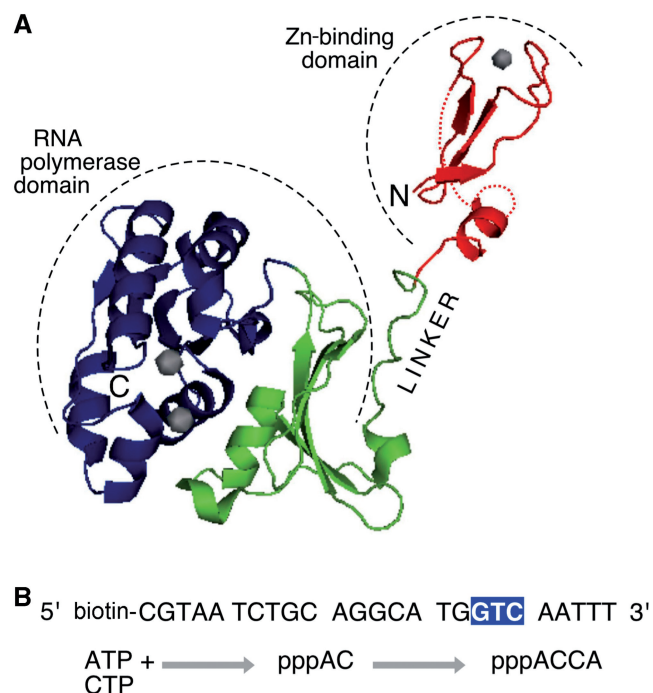


Figure 1. Organization of T7 DNA primase and template DNA used in this study. (A) The T7 DNA primase domain of gene 4 protein (residues 1–245) is composed of a ZBD (residues 1–54) and an RPD (residues 71–245) connected by a flexible linker region of 16 amino acid residues (6). The primase domain used in this study contains an additional 26 amino acid residues that, in the full-length gene 4 protein, connect the C-terminus of the primase to the helicase domain. (B) Sequence recognition and primer synthesis by T7 DNA primase. T7 primase recognizes a basic trinucleotide sequence (5'-GTC-3' shown in box) at which the primase catalyzes the synthesis of the dinucleotide pppAC. If the appropriate nucleotides are present in the template, predominately G and T, then the primase will extend the dinucleotide to the functional tetranucleotide, in this case, pppACCA. Note that the 'cryptic' cytosine is not copied into the primer but is essential for recognition.

extended by the primase to a tetranucleotide, a preformed tetranucleotide can be used by the polymerase to initiate synthesis. Alterations in the ZBD and RPD cause disruption in the sequence-specific template recognition and catalytic function, respectively, resulting in failures in primer synthesis (18,19,21,22). For example, alteration in the ZBD affects the ability of the protein to recognize the trinucleotide recognition site (21,22). Without the ZBD, random diribonucleotides are synthesized, albeit at a very low rate, in the presence or absence of DNA. In addition to many critical residues in the ZBD and RPD, the length of the linker connecting the ZBD and RPD is critical to the catalytic steps during primer synthesis (23). The flexible nature of the linker also allows the ZBD of one subunit of a gene 4 protein hexamer to interact with the RPD of an adjacent subunit to catalyze the synthesis of a tetranucleotide (24).

The DNA primases also maintain the stability of the short primer-DNA hybrid until it can transfer it to the proper DNA polymerase. The latter step where the primase must interact with the polymerase is particularly important since the primase-polymerase interaction

enables the polymerase to extend a considerably shorter oligonucleotide than it could in the absence of the primase. The mechanism by which the zinc motif of the primase recognizes a trinucleotide sequence in ssDNA has been difficult to dissect in the absence of a structure of the enzyme in complex with DNA. An answer to this question would provide valuable information on the usage of primase recognition sequences, primer utilization by the polymerase and the mechanism of regulation of lagging strand synthesis. Kinetic studies have previously shown that the affinity of the T7 DNA primase for its template is weak, ranging from 10 to 150 μM (25). The tight DNA binding (several nanomolar) observed with the full-length 63-kDa gene 4 protein occurs via interactions between ssDNA templates and the helicase domain.

In the present study, we have examined the interaction of the DNA primase encoded by phage T7 with its primase recognition sequence using a combination of surface plasmon resonance (SPR) and biochemical assays. The kinetic data mentioned above identify a weak interaction but the precise requirements for this interaction are not known. Since the kinetic studies measure the product of the reaction, it is not clear if the primase alone binds to its recognition site, if the nucleoside triphosphate precursors are required or the di- and trinucleotide intermediates or the final functional tetranucleotide should be formed. We have, for the first time, obtained quantitative information of the physical binding of the primase to its recognition sequence and the effect of NTPs on this binding. Based on the results, we suggest a model in which conformational changes induced during primer synthesis result in a stable and selective DNA-primase complex.

MATERIALS AND METHODS

Materials

Synthetic oligonucleotides were obtained from Integrated DNA Technology (DNA), Oligos etc. and Dharmacon (RNA). Nucleotides and column resins used for protein purification were purchased from GE Healthcare. Sensor chips and reagents for SPR studies were from Biacore (Uppsala, Sweden). A plasmid expressing the ZBD of T7 gene 4 protein was a generous gift from Masato Kato (Harvard Medical School).

Plasmid construction and protein purification

Plasmids expressing various forms of T7 DNA primase were constructed following a standard protocol described previously (6,12). Full-length primase contains the first 271 amino acid residues from the N-terminal of the gene 4 protein (residues 1–271). ZBD is the first 59 amino acid residues (residues 1–59) and RPD is a C-terminal portion of the primase (residues 64–271). Proteins were overproduced in *E. coli* strain HMS174(DE3). After the bacterial culture reached OD_{600} around 1, protein was induced with Isopropyl β -D-1-thiogalactopyranoside (IPTG) at the final concentration of 1 mM at 37°C for 3 h. The induction condition was modified to 5 h at room temperature in case a protein was not abundantly

overproduced. After harvest, the bacterial cells were ruptured by three cycles of freeze-thaw in the presence of lysozyme and the clear lysate containing overproduced protein was collected by centrifugation for the subsequent column chromatography. The full-length primases were purified with sequential column chromatography in the order of Diethylaminoethyl cellulose (DEAE) anion exchange, S-200HR gel filtration and blue affinity column chromatography as described previously (12). Purification for the RPD was carried out following the same protocol as described for the full-length primase except that the unbound flow-through fractions from the DEAE column chromatography was collected and used for subsequent steps. The ZBDs were purified using DEAE anion exchange chromatography followed by G-50 gel filtration chromatography (6). All proteins were further cleaned on Mono Q anion exchange column to ensure that proteins were free from any nucleases. After the final purification step, proteins were precipitated by ammonium sulfate and the resulting pellets were dissolved and dialyzed against a buffer (20 mM Tris-HCl, pH 7.5) before use.

SPR

SPR analysis was performed at room temperature using a Biacore-3000 instrument (Uppsala, Sweden). Indicated amounts of ssDNA biotinylated at the 5'-end were coupled to streptavidin-coated chip by flowing 20 nM DNA in a coupling buffer (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl and 1 mM Ethylenediaminetetraacetic acid (EDTA)) at a flow rate of 10 $\mu\text{l}/\text{min}$. Equal moles of biotin were also immobilized to surface of the chip in the reference flow cell to subtract the bulk refractive index of the buffer and the non-specific interaction with the surface. Binding of protein to the immobilized DNA was carried out in a binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM potassium glutamate, 10 mM MgCl_2 and 1 mM Dithiothreitol (DTT)) at a flow rate of 40 $\mu\text{l}/\text{min}$. The chip surface was regenerated by injecting 100 μl of 1 M NaCl and 50 mM NaOH at a flow rate of 100 $\mu\text{l}/\text{min}$ to remove bound proteins. The dissociation constant, K_D , was calculated by fitting the average Response unit (RU) to a steady state model provided by the BIAeval 3.0.2 computational software (Biacore).

Primer synthesis assay

The indicated amount of T7 DNA primase was incubated with 0.5 or 5 μM of 25-mer ssDNA template, 0.2 mM each of ATP and [α - ^{32}P] CTP (0.1 $\mu\text{Ci}/\mu\text{l}$) in a reaction buffer containing 40 mM Tris-HCl, pH 7.5, 50 mM potassium glutamate, 10 mM MgCl_2 and 10 mM DTT. The template either contains the cryptic cytosine (5'-TGGTC-3' or 5'-GGGTC-3') or not (5'-TGGTG-3') in the DNA sequence. After 10 min incubation at room temperature, the reaction was terminated by the addition of sequencing dye and the reaction products were separated on a 25% sequencing polyacrylamide gel containing 3 M urea. The gel was dried for autoradiography and the amount of Cytosine monophosphate (CMP) incorporated into a major product, tetranucleotide (pppACCA or

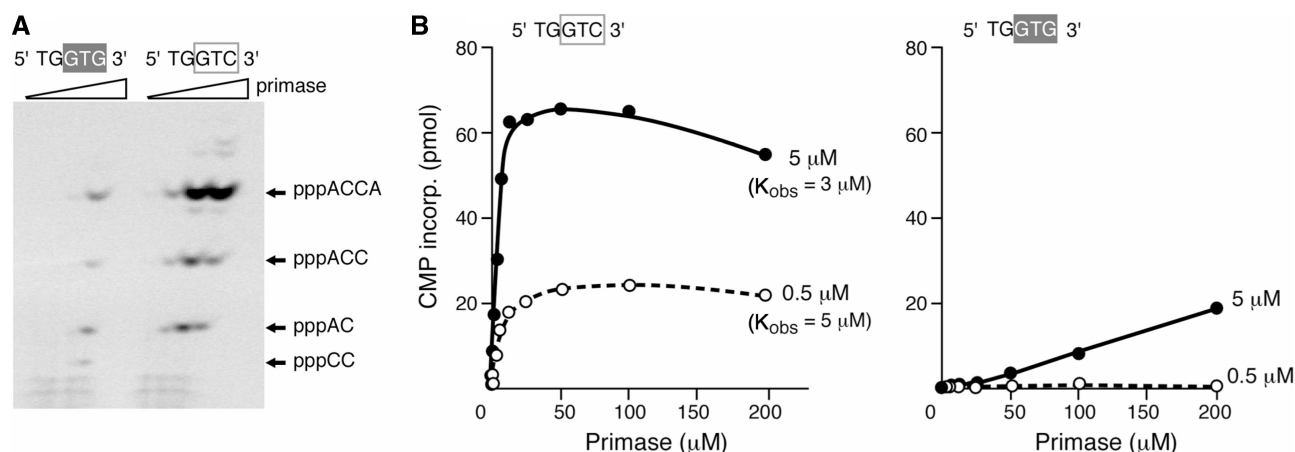


Figure 2. Sequence-specific synthesis of oligoribonucleotide by T7 DNA primase. (A) Template-directed oligoribonucleotide synthesis was carried out using the 25-mer template described in Figure 1B in which a primase recognition site is either present (5'-TGGTC-3') or not (5'-TGGTG-3'). The standard reaction contains 0.2 mM each of ATP and [α - 32 P] CTP (0.1 μ Ci/ μ l), 5 μ M of the indicated template and various concentrations of T7 DNA primase (0, 1, 10 and 100 μ M). After a 10-min incubation at room temperature, the reaction products were separated on a 25% denaturing polyacrylamide sequencing gel. Reaction products are indicated at the right side of the gel. (B) Quantitative analysis of primer synthesis reaction. Similar to the reaction described in (A), reactions were carried out using 0.5 or 5 μ M template DNA and incorporation of CMP into a major product of the reaction, pppACCA, was measured and plotted against primase concentration. Apparent dissociation constants (K_{obs}) calculated from duplicated experiments are shown.

pppACCC) was analyzed using Fuji BAS 1000 Bioimaging analyzer. Apparent dissociation constants (K_{obs}) were calculated by plotting the CMP incorporation against primase concentration using non-linear regression analysis.

RESULTS

The synthesis of oligoribonucleotides by T7 primase occurs by the condensation of ribonucleotides in a template-directed manner. Diribonucleotide (pppAC) synthesis is initiated at specific trinucleotide recognition sites, 5'-GTC-3', followed by its extension to a tetranucleotide (pppACCC, pppACCA or pppACAC) depending on the sequence of the recognition site. An early step must be the recognition of 5'-GTC-3' by the primase, but it is neither known if the primase first binds to ssDNA in search of this sequence nor if ATP, CTP or both nucleotides must be present. We have used SPR analysis to measure binding of the T7 DNA primase to an oligonucleotide containing the primase recognition sequence 5'-TGGTC-3' (Figure 1B). The basic recognition sequence 5'-GTC-3', shown in bold, and the two additional 5'-nucleotides, T and G that provide the template for tetra-ribonucleotide synthesis, are indicated. The recognition sequence has five additional nucleotides on the 3'-side since a minimum of one is required for optimal recognition (25). Fifteen nucleotides are located on the 5'-side and separate the recognition sequence from the 5'-biotin label used for immobilizing to the sensor chip. The same oligonucleotide in which the cryptic C in the recognition sequence has been replaced with G (5'-TGGTG-3') serves as a control since T7 DNA primase does not catalyze template-directed synthesis from such a sequence (21,25). In this study, we have used the primase domain of gene 4 protein lacking the helicase domain (12) to avoid any effect from the

helicase domain. The presence of the helicase domain would lead to extremely tight binding via the central channel of the hexamer (9,25), thus masking the presumably weaker binding of the primase domain. This primase domain, containing the first 271 amino acid residues of the gene 4 protein, has been shown previously to exhibit template-directed primer synthesis similar to the full-length protein (12).

Biochemical analysis of oligoribonucleotide synthesis by T7 DNA primase

The enzymatic properties of T7 DNA primase have been extensively characterized but usually under conditions where the DNA template is in considerable excess over the primase (25). Since SPR analysis is carried out with an excess of primase and a limited amount of DNA template, we first examined primer synthesis and template specificity under conditions that mimic those used in SPR analysis. Under these conditions, nanomolar concentration of the template is present with the primase in the micromolar ranges. Consistent with previous results, a comparison of two templates shows that the cryptic cytosine in the primase recognition sequence is critical for efficient primer synthesis (Figure 2). While the sequence containing the cryptic cytosine (5'-TGGTC-3') directs synthesis of primers proportional to the concentration of primase, replacement of the cryptic base with guanine (5'-TGGTG-3') results in a significant reduction in primer synthesis (Figure 2A). Although a small amount of primer synthesis is observed with the template lacking the cryptic cytosine, ~100-fold more primase is required to produce the equivalent amount of primers generated with the template containing the primase recognition sequence. Quantitative analysis of the reaction under steady state condition provides a K_{obs} of 3–5 μ M for the

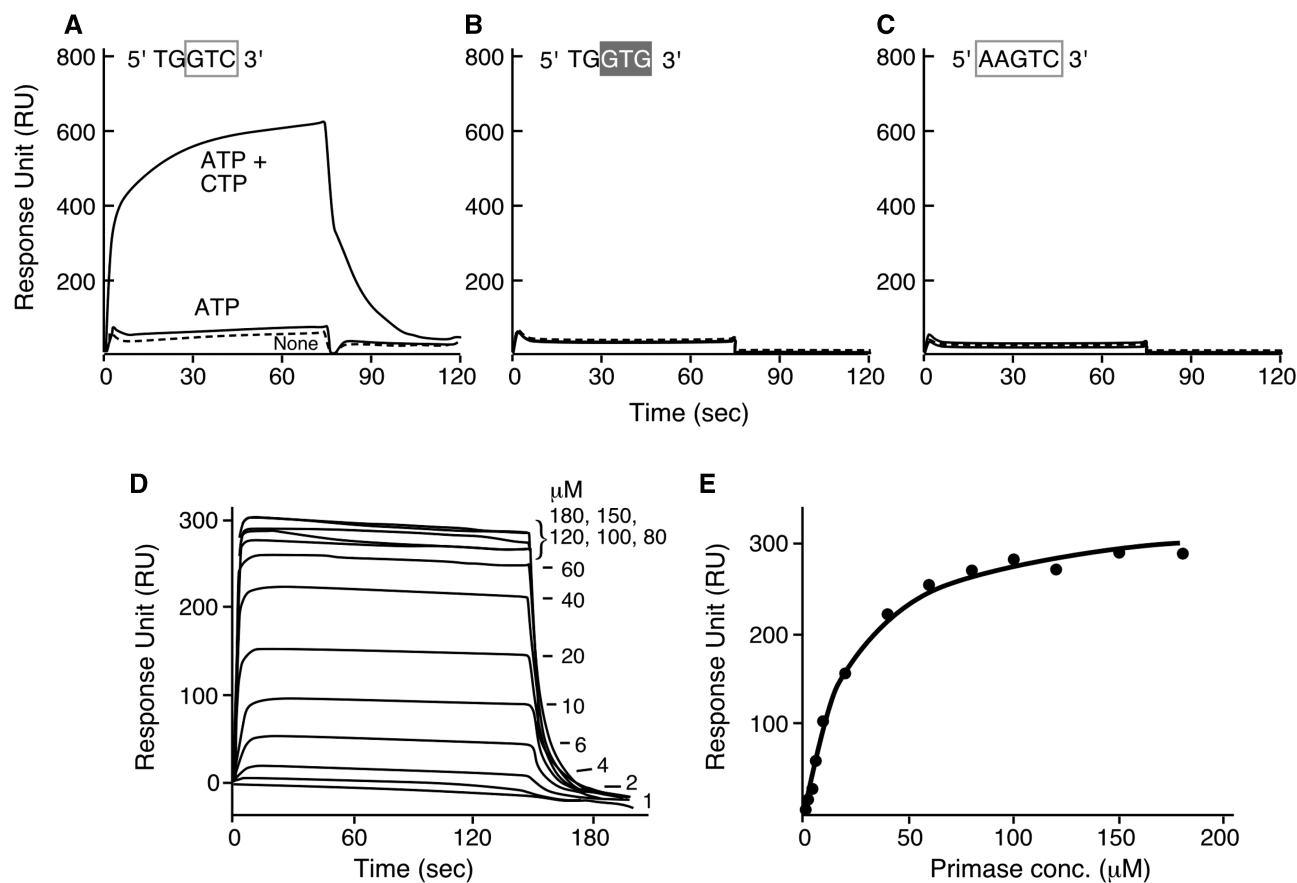


Figure 3. SPR analysis of binding of T7 DNA primase to its primase recognition sequence. (A–C) An equal amount (1000 RU, corresponding to 2 pmol) of a 25-mer ssDNA (see Figure 1B) containing variations of the T7 primase recognition sequence was immobilized on a Biacore sensor chip through a biotin–streptavidin interaction. The DNA strands contain template sequences for synthesis of tetranucleotide (5′-TGGTC-3′) or dinucleotide (5′-AAGTC-3′); a control sequence in which the cryptic cytosine is replaced with guanine (5′-TGGTG-3′) is also included. Primase (10 μM) was flowed over the chip together with the indicated components in a buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM potassium glutamate, 10 mM MgCl₂ and 1 mM DTT, and binding signals were detected. ATP and/or CTP were added at 0.2 mM. (D) Binding to the oligonucleotide containing the primase recognition site (5′-TGGTC-3′, 100 RU) in the presence of 0.2 mM each of ATP and CTP was measured at various concentrations of T7 DNA primase (1, 2, 4, 6, 10, 20, 40, 60, 80, 100, 120, 150 and 180 μM). (E) K_D of the primase binding to the recognition sequence in the presence of ATP and CTP. The dissociation constant (K_D) was calculated using the average RU obtained from (D) under steady state condition. The steady state RUs were plotted against the primase concentration and the data were fitted to a steady state model provided by the BIAeval 3.0.2 software (Biacore).

oligonucleotide containing the recognition sequence (Figure 2B). This value is lower than that previously found with excess template and a limited amount of primase (10–150 μM) (25), presumably due to more efficient primer synthesis by the excess primase used in the present experiments.

Sequence-specific binding of T7 DNA primase to ssDNA templates

An equivalent amount of ssDNA that either contains the cryptic cytosine or lacks the cryptic cytosine was immobilized on a sensor chip via a biotin–streptavidin interaction. Binding of the primase to the DNA was measured by flowing T7 DNA primase over the chip. Over a wide range of DNA immobilized (100–2000 RU), slightly better binding of the primase to the oligonucleotide containing the recognition sequence with the cryptic C (5′-TGGTC-3′) was observed (at most 100 RU higher) (data not shown). This slight preference, however, does

not explain the great specificity of the primase for its recognition sequence during primer synthesis as seen in biochemical assays. Since the primase catalyzes nucleotide polymerization in a template sequence-dependent manner, we examined its binding to DNA in the presence of nucleotides. The addition of ATP, the first nucleotide incorporated into the oligonucleotide during primer synthesis, does not stimulate the binding of primase to its recognition site (Figure 3A). Likewise, the presence of CTP, the second nucleotide for primer synthesis also does not change the binding pattern (data not shown). However, when both ATP and CTP are present, the binding of the primase to the oligonucleotide containing the primase recognition site is enhanced ~10-fold (Figure 3A). In contrast, the binding of the primase to the oligonucleotide lacking the cryptic cytosine is not affected by the addition of the NTPs (Figure 3B). These results clearly demonstrate that the presence of the NTPs significantly increases the binding affinity of the primase to

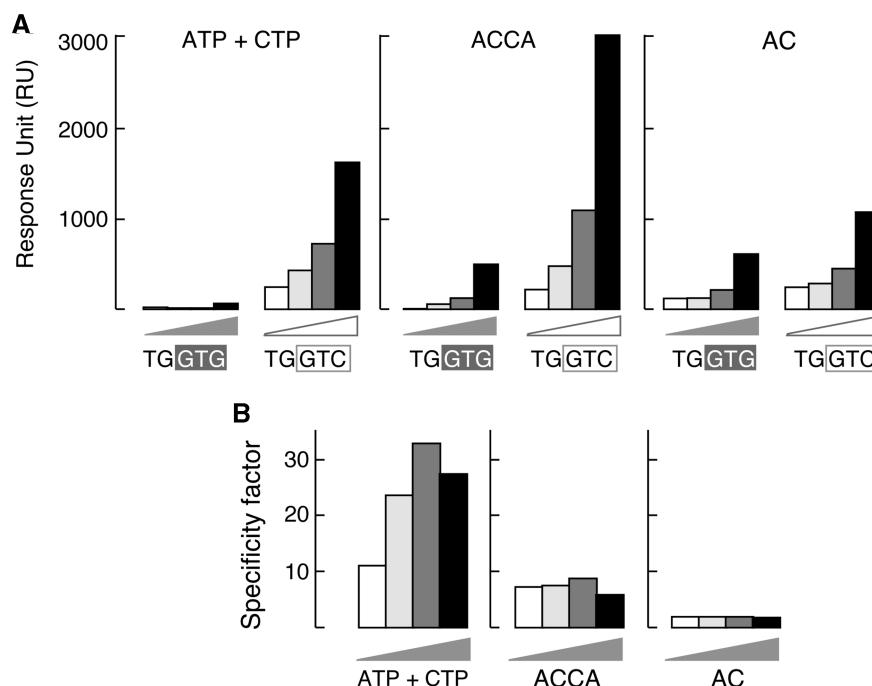


Figure 4. Sequence-specific binding of T7 DNA primase in the presence of preformed oligoribonucleotides. (A) Similar to Figure 3A and B, oligonucleotides (1000 RU) containing the primase recognition sequence or a sequence lacking the cryptic cytosine were immobilized and the primase (10 μ M) was flowed over the chip in the presence of nucleotides or preformed oligoribonucleotides. Bars indicate responses obtained in the presence of the nucleotides or oligonucleotides at the concentration of 20, 50, 100 and 250 μ M. (B) Template sequence-specific binding of the primase. Specificity factor was calculated by dividing the response obtained with the primase recognition sequence by the response obtained with the sequence lacking the cryptic cytosine in (A). Specificity factor of 1 represents no preferred binding of the primase to the oligonucleotide containing a primase recognition site, 5'-TG GTC-3'.

ssDNA only when the template contains a primase recognition sequence. We determined the dissociation constant (K_D) for primase binding to its recognition site in the presence of ATP and CTP by measuring binding at various concentrations of primase (Figure 3D and E). The maximum binding of primase calculated under this condition (375 RU, R_{max} = (molecular weight of the primase, 30 kDa)/(molecular weight of the 25-mer DNA, 8 kDa) * R_1 (RU from the immobilized DNA, 100 RU) * N (binding ratio of the primase to the immobilized DNA, assumed $N = 1$) corresponds to the experimentally obtained values (300 RU), indicating that the primase binds to the 25-mer ssDNA with a stoichiometry of 1:1. Fitting of the average steady state RUs to a steady state model yields a K_D of $22 \pm 1.8 \mu$ M.

It is difficult to address the question as to the basis of the enhancement of primase binding to its recognition sequence by ATP and CTP. The enhancement could arise from these nucleotides residing in their respective binding pockets while contacting the complementary base in the template. Alternatively, the ongoing synthesis of the primer or the complete primer itself could stabilize the primase at the site where synthesis is occurring. The initial step in primer synthesis is the formation of pppAC. T7 primase synthesizes the dinucleotide pppAC quite efficiently on oligonucleotides containing only this trinucleotide sequence such as 5'-AAGTC-3' (26). Oligonucleotides containing this abbreviated recognition sequence do not enhance the binding of DNA

primase to the DNA in the presence of ATP and CTP (Figure 3C). This result clearly shows that neither the simple binding of NTPs to their binding pocket nor the diribonucleotide formation is sufficient to enhance the primase binding.

Binding of T7 DNA primase to template DNA in the presence of preformed oligoribonucleotides

The results described above suggest that the presence of both ATP and CTP increases the binding of the primase to its recognition sequence by synthesizing the tetranucleotide pppACCA. To examine this possibility further, primase binding was measured in the presence of the preformed tetranucleotide ACCA. We also examined the effect of the diribonucleotide AC, an intermediate for tetranucleotide synthesis, on primase binding (Figure 4A). Both of these oligonucleotides enhance binding in a concentration-dependent manner. The tetranucleotide enhances primase binding to its recognition site ~ 2 -fold better than do ATP and CTP. However, unlike ATP and CTP, the preformed ACCA also significantly increases the binding of the primase to DNA lacking the primase recognition site. The preformed dinucleotide is not nearly as efficient in promoting binding of the primase to its recognition sequence, $\sim 60\%$ as efficient at the highest concentration of NTPs. Furthermore, the dinucleotide also enhances the binding of the primase to DNA lacking the recognition sequence even better than

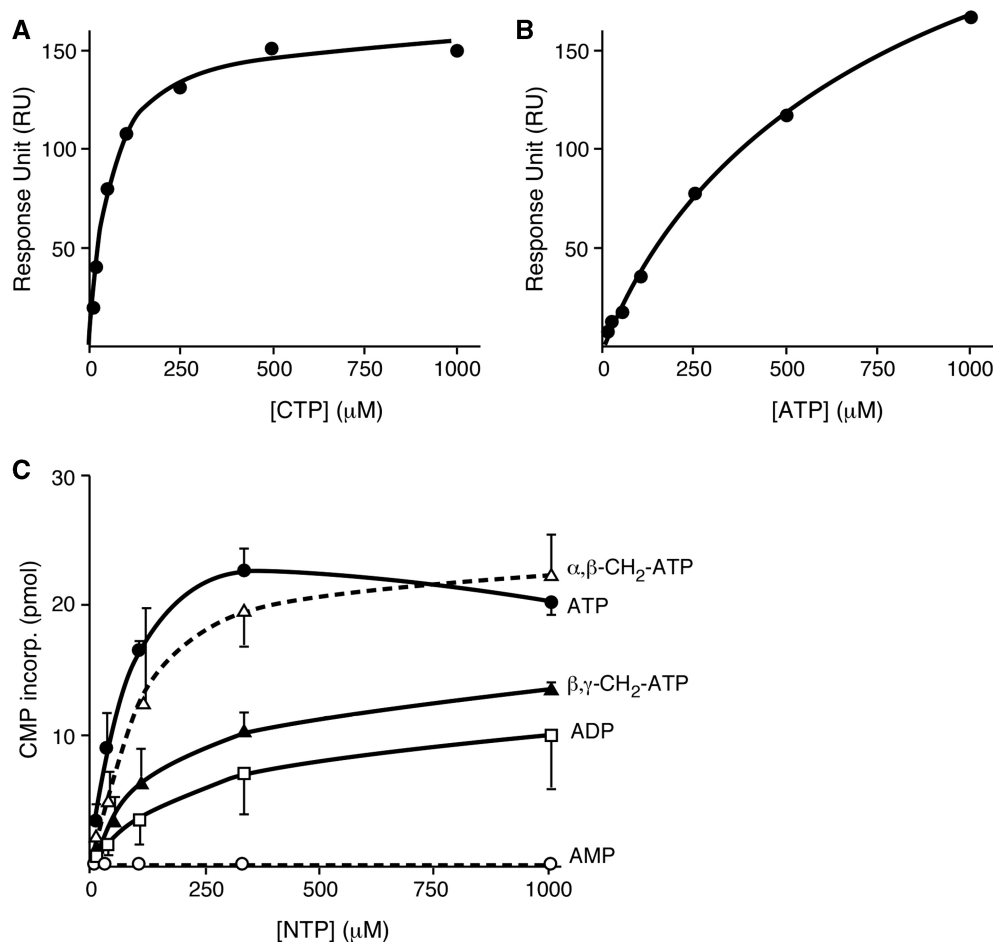


Figure 5. Effect of ATP and CTP on the binding of T7 DNA primase to its recognition sequence. Binding of the primase (10 μM) to an oligonucleotide containing a recognition sequence 5'-TG $\overline{\text{GTC}}$ -3' (250 RU) in the presence of various concentrations of ATP or CTP was measured by SPR assay. (A) The indicated concentration of CTP was present in the binding reaction together with 0.2 mM ATP. (B) The indicated concentration of ATP was present in the binding reaction together with 0.2 mM CTP. (C) Effect of the 5'-phosphate of ATP on primer synthesis. Primer synthesis reactions were carried out with 0.5 μM 25-mer ssDNA containing a recognition sequence 5'-GG $\overline{\text{GTC}}$ -3', 5 μM T7 DNA primase, 0.2 mM [α -³²P] CTP (0.1 μCi/μl) and various concentration of ATP or its analogs (12, 37, 111, 333 and 1000 μM). After separation of reaction products by polyacrylamide gel electrophoresis, incorporation of CMP into a major product, pppACCC, was determined by phosphorimager analysis and plotted against ATP or its analog concentration. Data from three independent experiments are shown.

does the tetranucleotide. The preference for the recognition sequence expressed as a specificity factor clearly indicates that NTPs are the most selective in enhancement of the primase binding to its recognition site followed by the preformed tetraribonucleotide; the diribonucleotide exhibits almost no selectivity (Figure 4B). Tetraribonucleotides synthesized *de novo* by the primase (pppACCA) bear a triphosphate at their 5'-terminus. A primer containing a single 5'-phosphate enhances binding of the primase to its recognition site somewhat better than does a primer lacking a 5'-phosphate (Supplementary Figure S1).

Effect of NTP on template binding by T7 DNA primase

As shown in the previous section, the presence of both ATP and CTP is pivotal for binding of the primase to DNA containing a primase recognition site. Examination of the effect of ATP and CTP concentration on the binding of primase to its recognition site supports a

distinct binding for CTP and ATP (26). CTP enhances the binding of the primase, in the presence of ATP, with a dissociation constant of 0.055 mM whereas the enhancement by ATP, in the presence of CTP, does not reach saturation even at 1 mM (Figure 5A and B). Since significant binding of the primase does not occur in the absence of either ATP or CTP, the NTP not being examined was present at 0.2 mM.

The role of ATP and CTP in the binding of primase to its template was further investigated by measuring the ability of NTP analogs to stimulate the binding using SPR analysis (Table 1). Any modifications on the ribose or phosphate groups in either nucleotide result in decreased binding of the primase. The absence or decrease in the binding of the primase in the presence of NTP analogs is consistent with their inability to support the synthesis of tetraribonucleotide (26). In order to confirm that defects in the binding also affect primer synthesis, primer synthesis with the ATP analogs was

determined. In this assay, we used a template DNA with the recognition sequence 5'-GGGTC-3' so that the adenosine nucleotide would only be incorporated into the first position of the primer. Examination of the efficiency in the tetranucleotide synthesis (pppACCC) shows that elimination of either the 2'- or 3'-hydroxyl group from the ribose abolishes primer synthesis (data not shown). The result obtained with the 3'-dATP is not surprising since it has no 3'-hydroxyl to carry out a nucleophilic attack on the CTP to synthesize the dinucleotide. A less but significant reduction was also observed as the 5'-phosphate groups of ATP were removed (ATP, ADP and AMP in Figure 5C). This result is in agreement with the better binding observed in the presence of a primer

Table 1. Effect of various nucleotides on binding of T7 DNA primase to its recognition sequence

Sequence in template	5'-TGGTG-3' Response units	5'-TGGTC-3' Response units
Nucleotide		
None	0 ± 3	0 ± 4
ATP + CTP	6 ± 2	665 ± 46
2'-dATP + CTP	-6 ± 10	3 ± 10
3'-dATP + CTP	-2 ± 7	49 ± 14
ddATP + CTP	-3 ± 7	15 ± 2
AMP + CTP	-5 ± 2	10 ± 2
ADP + CTP	3 ± 4	58 ± 2
α,β-methylene ATP + CTP	-4 ± 7	274 ± 17
β,γ-methylene ATP + CTP	10 ± 13	57 ± 27
ATP + 2'-dCTP	-5 ± 3	-8 ± 7
ATP + 3'-dCTP	-4 ± 4	2 ± 6
ATP + ddCTP	-4 ± 3	-12 ± 4
ATP + CMP	-7 ± 3	-12 ± 5
ATP + CDP	-7 ± 1	97 ± 17

DNA templates (550 RU) containing the indicated sequence were immobilized on a Biacore chip as described in 'Materials and Methods' section. T7 DNA primase (10 μM) was flowed over the chip in the absence or presence of the indicated nucleotide at the final concentration of 0.2 mM each. Response units were normalized against the values obtained in the absence of nucleotide. Data were obtained from duplicated trials.

containing one phosphate relative to the unphosphorylated primer (Supplementary Figure S1). A non-hydrolyzable analog, α, β-methylene ATP can be incorporated into the primer as efficiently as ATP but β,γ-methylene ATP is less effective. These results suggest that interactions involving the 5'-phosphates of ATP within the ATP binding pocket are important parameters for primer synthesis.

Binding of T7 DNA primase subdomains to template DNA

T7 DNA primase consists of a ZBD and RPD that are connected by a flexible linker (Figure 1A). For primer synthesis, the ZBD of T7 DNA primase must recognize specific sequences in the template and the RPD needs to contact the DNA template for catalysis of NTP condensation. To determine the contribution of each of these subdomains to DNA binding, we have examined the binding of the ZBD (residues 1–59) and the RPD (residues 64–271), each purified separately from the other. In contrast to the entire primase domain, neither the ZBD nor the RPD alone exhibit detectable binding to oligonucleotides containing the recognition sequence in the presence of both ATP and CTP (Figure 6A and B). The addition of the preformed tetranucleotide ACCA does not increase the binding of the subdomains (data not shown). The results suggest that the stable binding of the primase to its recognition requires interaction between the two subdomains. Although a mixture of the separate ZBD and RPD can catalyze synthesis of oligonucleotides at low efficiency (27), this temporary association is not sufficient to produce stable binding as measured by SPR (data not shown).

Since neither the ZBD nor the RPD binds to DNA containing the recognition sequence, we examined binding of less drastically altered primase in which both subdomains are present but a single amino acid substitution eliminates the function of the RPD. Lys122 in the RPD is involved in NTP binding and replacement of Lys122 with an alanine abolishes catalytic activity of the

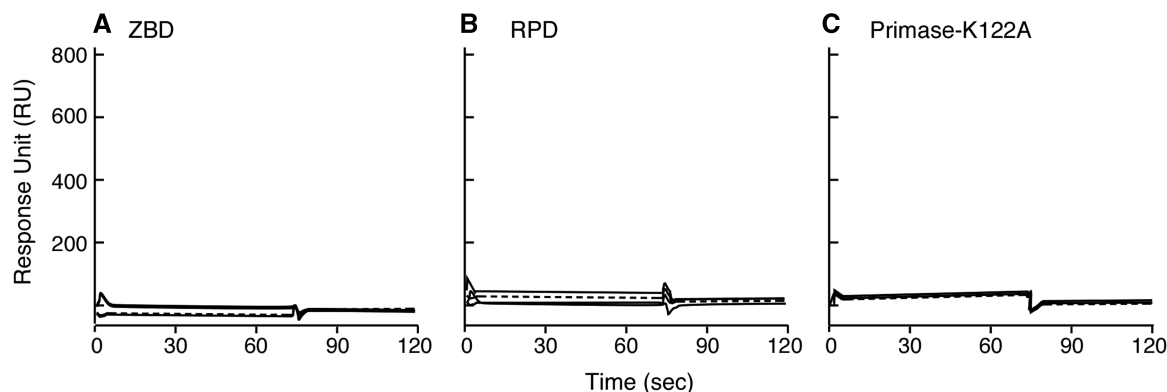


Figure 6. Binding of T7 DNA primase subdomains and altered primases to DNA. Similar to the conditions described in Figure 3, oligonucleotide (1000 RU) in which a primase recognition site is present (5'-TGGTC-3') or not (5'-TGGTG-3') was immobilized and binding of the primase was monitored in the absence or presence of 0.2 mM each of ATP and CTP. ZBD (residues 1–59) and RPD (residues 64–271) are the purified fragments containing the ZBD and RPD of the T7 DNA primase, respectively. Primase-K122A is an altered T7 DNA primase containing the indicated single amino acid substitution K122A in the RPD. Proteins were flowed over the chip at the concentration of 10 μM.

primase (primase-K122A) without changing its specificity for sequence recognition (18). Examination of primase-K122A revealed no detectable binding to any template in the absence or presence of NTPs (Figure 5C).

DISCUSSION

T7 DNA primase catalyzes the template-directed synthesis of oligoribonucleotides at specific sequences on ssDNA for subsequent use by DNA polymerase to initiate DNA synthesis (3). However, insight into the mechanism by which the primase recognizes these sequences has been elusive. In the prokaryotic DNA primases, the ZBD is clearly involved in this recognition but it alone does not appear to be sufficient. Therefore, the RPD of the primase must be involved. The RPD is tethered to the ZBD by a flexible linker, suggesting that considerable rearrangements of the primase must occur during recognition of the proper sequences and following nucleotide condensation. In the past, attempts to demonstrate physical binding of the primase to its recognition sequences have been unsuccessful, the only binding data coming from kinetic studies where the primase exhibited low affinity for its recognition sites (10–150 μ M) (25). In the present study, we show that the presence of NTPs required for the synthesis of the tetranucleotide primer is necessary to obtain significant binding of the T7 DNA primase to DNA containing its recognition sequence. NTPs are more efficient than preformed oligoribonucleotides complementary to the recognition site in selective binding to the template containing the primase recognition sequence. Loss of sequence-specific DNA binding by amino acid changes introduced into the catalytic subdomain of the primase support the requirement for synthesis of a tetranucleotide for maximum binding.

Interactions that facilitate DNA primase binding to its recognition sequence

A striking observation is the large enhancement in the binding of T7 primase to DNA containing its recognition sequence by the combination of ATP and CTP. The increased binding must occur through the binding of the NTP to the catalytic sites of the RPD and subsequent primer synthesis. While the dinucleotide pppAC generated from the initial condensation of ATP and CTP is not sufficient, the functional tetranucleotide produced through the subsequent extension stabilizes the template–primase complex. Since the ZBD is clearly involved in template recognition (15), there must be interplays between the primase recognition sequence, the NTPs, the subsequently synthesized primer and the two subdomains in the primase. Earlier experiments suggest that such interactions are important parameters contributing to conformations of the primase that lead to primer synthesis. For example, oligoribonucleotide synthesis is modulated by the length of the linker connecting the ZBD and RPD, lengths that might lead to different contacts between the subdomains (23). NMR studies indicate that the addition of a primer-template induces its interactions with the ZBD and RPD, both of which

were not associated each other in the absence of the template (6). Structural and biochemical studies on *E. coli* DnaG primase also suggest that the ZBD and RPD communicate with one another to regulate primer synthesis (28). Therefore, the role of the ZBD is not limited to recognition of a specific template sequence but can be extended to stabilization of the primase–DNA complex through critical interactions with the RPD.

Loss of DNA binding in an altered T7 primase containing a defective RPD (primase-K122A) is most likely due to a defect in the binding of ATP or CTP (19). In any case, the consequence is the inability of the primase to catalyze the synthesis of even the first dinucleotide.

NTP binding sites of T7 DNA primase

It has been proposed that T7 DNA primase has two separate NTP binding sites: one for ATP and one for CTP (3). T7 primase can catalyze the synthesis of random dinucleotides in the absence of a recognition sequence or even in the absence of DNA but synthesis is extremely slow (15,27). Interactions between the ZBD and RPD facilitate the condensation of the first two ribonucleotides and further extension into tetranucleotide. The two NTP binding sites appear to be structured specifically for ATP and CTP (Figure 5) (19,21). It is interesting that most DnaG family DNA primases selectively incorporate ATP at the first position (3). Not only the number but also the flexibility of the phosphate group of the first NTP is important for binding, consistent with previous data (20). Examination of templates containing a repeated sequence of any two base combinations for primer synthesis reveals that T7 primase can also synthesize (AG) $_n$ as well as (AC) $_n$ at high concentrations (Seung-Joo Lee and Charles C. Richardson, unpublished data). Although the biological relevance for the two repeat primers remains elusive at this point, it suggests that the second binding site has some level of plasticity to adapt to nucleotides other than CTP.

Stepwise analysis of sequence-specific template recognition by DNA primase

Based on our results, the sequence-specific recognition by T7 DNA primase can be divided into several steps (Figure 7). The first step is binding of ATP and CTP to their respective sites in the RPD (Stage I). Although a minimal level of dinucleotide synthesis is observed in the absence of either the ZBD or ssDNA (15), the most efficient template-directed condensation of NTPs occurs only when the ZBD recognizes a template containing the trinucleotide sequence with the cryptic cytosine (21) (Stage II). The template-directed dinucleotide formation requires contact of the basic recognition sequence 5'-GTC-3' by both the ZBD and RPD. Therefore, conformational changes in the primase that facilitate interactions of both subdomains with the recognition site are expected. Nonetheless, dinucleotide formation does not increase the binding of the primase to the DNA (Figure 3), clearly showing that the binding we observed is not accounted for by their synthesis from ATP

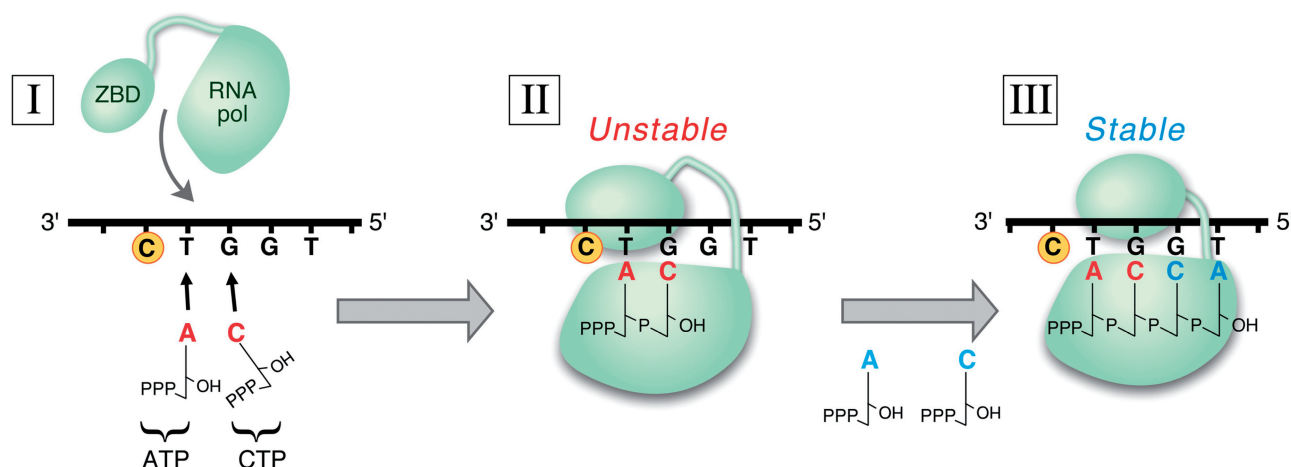


Figure 7. Model for binding of T7 DNA primase to template containing a primase recognition sites. Stepwise analysis of the primase binding to template DNA is described in the 'Discussion' section.

and CTP. The accumulation of a constant amount of diribonucleotide is detected during synthesis of primers longer than dimer, supporting the model that the dimer is an abortive product and that the rate for its formation is faster than that for its extension (25). The diribonucleotide has to be extended to the functional tetranucleotide in subsequent steps. The fact that a shorter linker connecting the ZBD and RPD facilitates the extension to tetranucleotide suggests that the primase undergoes another conformational shift that establishes different contacts between the subdomains (23). For the third and fourth positions of the tetranucleotide, the two NTP binding sites now can accept ATP or CTP depending on the template sequence to synthesize pppACCC, pppACCA or pppACAC (25). Whether the ATP in the extension reaction binds to the initial ATP binding site used for the initiation of primer synthesis or simply binds to the CTP site is not known. Only after tetranucleotide formation does the primase form a stable complex with the primer and the recognition sequence (Stage III).

The preformed tetranucleotides complementary to the recognition sequence can also increase the primase binding by maintaining necessary interactions with the primase and template containing a recognition sequence. However, compared with NTPs, the preformed tetranucleotide is less stringent for DNA sequence in complex formation with the primase (Figure 4). In order to form the stable complex with template containing a primase recognition sequence, the primase has to undergo at least two conformational changes: one during diribonucleotide formation (Stage II) and another one during its extension to tetranucleotide (Stage III). The preformed primer does not distinguish a primase recognition site as efficiently as does the oligonucleotide synthesized *de novo*, presumably because it does not induce such conformational changes in the primase. Therefore, we propose that conformational changes induced during both diribonucleotide formation (Stage II) and its extension to tetranucleotide (Stage III)

are key factors for a stable and specific primase complex formation.

The relatively weak binding hampers detection of a stable primase–template complex by DNA foot-printing analysis. However, a careful examination employing chemical cross-linking, followed by exonuclease digestion, does reveal a sequence-specific and NTP-dependent complex of primase and template (Supplementary Figure S2).

Comparison of DNA primase with RNA polymerase

Both DNA primases and RNA polymerases catalyze the template-directed synthesis of RNA. The family of DnaG primases share several mechanistic features with prokaryotic RNA polymerases. First, both enzymes recognize specific sequences in DNA from which they initiate synthesis of RNA. While the ZBD of prokaryotic DNA primases recognizes specific trinucleotide sequences in ssDNA template, the N-terminal domain of RNA polymerase (or σ factor) is responsible for detection of promoter sequences in dsDNA. Second, both enzymes undergo conformational changes during product formation. T7 RNA polymerase has to undergo transition from an unstable initiation stage to a stable elongation stage at which point it achieves high processivity (29). Before the transition, the unstable abortive initiation stage of the enzyme produces short RNA products (8–10 nt). Similarly, T7 DNA primase requires a transition from diribonucleotide formation to an extension mode to synthesize functional primers. Failure of such a transition to occur yields diribonucleotide (20,30). Third, despite disparity in overall structures, both have a concave active site together with an N-terminal regulatory domain. The N-terminal domain of T7 RNA polymerase confers its unique ability to recognize specific promoter sequences, melt dsDNA and initiate the synthesis of RNA without a primer, features distinct from other those of members of the pol I family (31). As described earlier, interactions between the ZBD and the RPD in T7 DNA primase are

essential for specific recognition and synthesis of oligoribonucleotides. An additional role of the ZBD in delivery of synthesized primers to DNA polymerase has been demonstrated previously (18). Obvious differences between the two enzymes such as the mode of synthesis (processive versus diffusive), the type of DNA template and the mechanism for sequence recognition suggest that the two enzymes are evolutionarily diverged. However, the ability of RNA polymerases to initiate replication in some plasmids and bacteriophages makes it reasonable to consider that DNA primases have evolved from RNA polymerases (32).

Contribution of sequence-specific DNA binding by DNA primase to replication

Binding of the primase to its recognition sequence has in the past not been considered a major player in the overall binding of the primase to DNA. *E. coli* DNA primase binds with relatively high affinity to DNA in the absence of its cognate helicase (33). Nevertheless, it does function in association with the DnaB helicase, an association that may provide for additional binding affinity. T7 primase is covalently linked to the DNA helicase. The tight binding of the helicase to ssDNA and its ability to translocate on ssDNA provide a major determinant for the association of the T7 primase to DNA. However, since the outside of the helicase domain is predominantly negatively charged (9), the helicase domain does not appear suitable for the initial contact with DNA, a prerequisite to its assembly onto the DNA. On the other hand, multiple primase domains flexibly anchored to the hexameric gene 4 protein are positioned to interact with ssDNA (16,34). This arrangement raises the possibility that a basic cleft in the RPD of the primase, a postulated ssDNA binding site (4,6), would be the initial binding site to DNA. A flexible linker connecting the primase and helicase domain might be advantageous to transport the DNA strand bound to the primase into the central channel of oligomeric helicase. Such an initial contact might facilitate rearrangement or ring opening of the oligomeric gene 4 protein for DNA binding (35). Our earlier studies have shown that in the absence of DNA the oligomeric gene 4 protein consists of a mixture of hexamers and heptamers. However, only the hexamer is found bound to DNA, giving rise to a model whereby loss of a subunit from the heptamer leads to ring opening and loading onto ssDNA (36).

Our preliminary examination indicates that gene 4 protein also binds preferentially to the primase recognition site similar to that observed with the primase domain. These studies are complicated by the fact that binding of the helicase domain to ssDNA in the presence of dTTP, the preferred nucleotide for oligomerization and translocation on DNA, masks the contribution of binding via the primase to its recognition site. Gene 4 protein does not show preferential binding to DNA containing a primase recognition sequence in the presence of dTTP. However, when both ATP and CTP are present together with dTTP, the overall DNA binding level is decreased, yet preferential binding to the primase recognition site is retained (Seung-Joo Lee and Charles C. Richardson,

unpublished data). This result suggests that binding of gene 4 protein through its primase domain can affect DNA binding by the helicase domain. More important, the sequence-specific binding of T7 DNA primase is observed with not only ssDNA but also dsDNA, extending the roles of sequence-specific binding by the primase in replication (Supplementary Figure S3). This observation is intriguing in that sequence-specific binding of dsDNA by many proteins containing a ZBD does not require the presence of NTPs. Yet it raises the possibility of an additional role of DNA primase such as recognition of the replication origin. In fact, the primary origin of T7 DNA replication contains a single primase recognition sequence (37). We propose that the primase domain is an initial loading site for the gene 4 protein to bind DNA and that both the primase domain and the helicase domain reciprocally influence each other for DNA binding (38). This model is similar to the one proposed earlier based on pre-steady state kinetics (35).

The stable complex formation of the primase with template containing a primase recognition site, observed in this study, is significant in the regulation of the replisome. If the primase remains bound to its recognition sequence after the *de novo* synthesis of primers, it would influence lagging strand synthesis by modulating primase recognition site usage. For instance, the primase in complex with a primer cannot recognize other primase recognition sites generated by the helicase, thus limiting the usage of primase recognition sites. The stabilization of the tetranucleotide by the primase is also required for its eventual transfer to a lagging strand DNA polymerase. The association could also result in conformational changes in the helicase domain, perhaps halting leading strand synthesis during primer hand-off to the lagging strand polymerase as detected in a form of transient pausing of leading strand synthesis in the presence of ATP and CTP (13). A recent report (39) demonstrated that T7 DNA primase remains bound with primer/template until it hands off the synthesized primer to DNA polymerase by forming a priming loop. In either case, the stable and sequence-specific primase-primer/template complex demonstrated in this study provides a molecular basis for the regulation of dynamics within the replisome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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